Plastid rRNA Operon Promoter Elements for Construction of Chimeric Promoters for Transgene Expression

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This application claims priority to US Provisional Application 60/433,302, filed December 13, 2002. The entire disclosure of the '302 application is incorporated by reference herein.

Pursuant to 35 U.S.C. §202(c), it is acknowledged that the U.S. Government has certain rights in the invention described, which was made in part with funds from the National Science Foundation, Grant Number MCB 99-05043.

Field of the Invention

This invention relates to the fields of molecular biology and the creation of transgenic plants. More specifically, the present invention provides novel Prrn based promoter constructs useful for driving high level expression of heterologous proteins in plastids.

Background of the Invention

Several patent documents and research articles are cited throughout this application in order to more fully describe the state of the art to which this invention pertains. The disclosure of each of these citations is incorporated by reference herein.

The most abundant transcripts in plastids are the ribosomal RNAs (rRNAs). The biosynthesis of plastid rRNA is highly regulated during development at both the transcriptional and posttranscriptional levels. In barley, rates of *rrn* transcription vary by 50-fold and

rRNA stability by 35-fold in response to developmental and environmental cues (Baumgartner et al., 1993). Rates of *rrn* transcription were induced 10-fold in pea and tobacco chloroplasts in response to light (DuBell and Mullet, 1995; Shiina et al., 1998; Chun et al., 2001). Transcription of the plastid ribosomal RNA operon (*rrn*) in higher plants is from diverse promoters. The *rrn* operon in tobacco is transcribed by the multisubunit, plastid encoded RNA polymerase (PEP) from a sigma-70 type promoter (PrrnP1)(Vera and Sugiura, 1995)as in most higher plants including maize (Strittmatter et al., 1985), pea (Sun et al., 1989), carrot (Manna et al., 1994), rice (Silhavy and Maliga, 1998), barley (Hubschmann and Borner, 1998) and Arabidopsis (Sriraman et al., 1998a). In tobacco, in addition to the PrrnP1 PEP promoter, *rrn* is transcribed from a second promoter, PrrnP2, recognized by the nuclear-encoded plastid RNA polymerase (NEP) (Vera and Sugiura, 1995; Allison et al., 1996). In spinach, transcription of *rrn* initiates in the same region, but from a promoter distinct from the PrrnP1 or the PrrnP2 promoters. This promoter, Pc, is the only promoter upstream of the *rrn* operon in spinach and is probably also recognized by the NEP (Iratni et al., 1997; Bligny et al., 2000). Pc is utilized as a second *rrn* promoter in Arabidopsis (Sriraman et al., 1998a), and is recognized in mustard chloroplasts (Pfannschmidt and Link, 1997).

To identify promoter elements important for PrrnP1 function, promoter dissection was carried out *in vivo* and *in vitro*. *In vivo* dissection was carried out by studying expression of *uidA* reporter genes from an ordered set of PrrnP1 promoter derivatives (Staub and Maliga, 1993; Allison and Maliga, 1995). *In vitro* dissection was carried out by measuring transcript accumulation from mini-genes which consist of a PrrnP1 promoter derivative

and transcription terminators (Jolly and Bogorad, 1980; Link, 1984; Gruissem and Zurawski, 1985; Orozco et al., 1985). *In vivo* dissection of the plastid *rrn* operon promoter indicates that sequences upstream of the conserved -35 box are important for promoter function.

Summary of the Invention

It is an object of the invention to identify and characterize promoter sequence elements which are responsible for the high transcription level of the plastid rnn operon. Such sequence elements are useful for the construction of chimimeric promoters for driving transgene expression from the plastid genome. Also disclosed are novel, isolated Prnn promoter derivatives that are useful for driving the expression of transgenes. The Prnn promoter derivatives can be used to advantage to enhance or decrease expression levels of heterologous molecules relative to that observed using a wild type Prnn promoter.

In one embodiment, an isolated nucleic acid sequence for promoting expression of heterologous molecules in the plastids of higher plants selected from the group of Prrn derivative sequences having SEQ ID NOS: 4-30 are provided. Such promoter derivatives can be operably linked to a sequence encoding a heterologous molecule of interest or precursor thereof. Heterologous molecules of interest include proteins and RNAs, such as siRNA. Vectors comprising the aforementioned chimeric sequences are also encompassed by the invention. In a preferred embodiment, a transgenic plant comprising such a vector is provided.

In yet another embodiment of the invention, an isolated nucleic acid sequence for promoting expression of heterologous molecules in the plastids of higher plants comprising mutations which mimimize homologous

recombination at the Prrn operon having the sequence of SEQ ID NO: 51 is disclosed. As above, this sequence can be operably linked to a sequence encoding a heterologous molecule of interest or precursor thereof. Also encompassed by the invention are vectors suitable for expression in plastids comprising SEQ ID NO: 51 operably linked to a sequence encoding a heterologous molecule of interest. Finally, transgenic plants comprising such a vector are within the scope of the present invention.

In another aspect, a chimeric promoter for expression of transgenes in the plastids of higher plants, comprising at least one Prrn transcription modulating element (PTME) operably linked to a core promoter selected from the group consisting of those provided in Table I are provided. In preferred embodiments, the PTME is SEQ ID NO: 50 and the promoter elements are either rbcl and or psbD. Such chimeric promoters are further operably linked to a sequence encoding a heterologous molecule of interest. Vectors and plants comprising the aforementioned constructs are also within the scope of the invention.

Brief Description of the Drawings

Figure 1 shows the alignment of the tobacco plastid (*Nt*) (SEQ ID NO: 1) and *E. coli* (*Ec*) (SEQ ID NO: 2) rRNA operon upstream regions. Nucleotide position is given relative to the transcription initiation site (TIS; +1; horizontal arrows). The Fis binding sites (bold), and the promoter recognition region, including the UP element and the conserved -35 and -10 elements, are marked. For tobacco, the 3' end of *trnV* gene is also indicated.

Figures 2A, 2B and 2C show the strategy used for identification of PrrnP1 Upstream Promoter. Fig. 2A: Tobacco PrrnP1 promoter deletion derivatives. Nucleotide

position is given relative to the transcription initiation site (+1; horizontal arrows). The position of the conserved -35 and -10 elements are marked. Plasmid names are listed on the left. Fig. 2B: Plastid transformation vector with Left and Right targeting sequences, the selectable marker (*aadA*) and *uidA* reporter gene. Position of plastid genes *rrn16*, *trnV* and *rps12/7*, and relevant restriction sites is indicated. Horizontal arrows mark gene orientation. Fig. 2C: RNA gel blot to test steady-state levels of *uidA* mRNA in transplastomic plants. Probing for the cytoplasmic 25S rRNA is used as the loading control.

Figures 3A, 3B and 3C show the strategy employed for identification of PrrnP1 Upstream Promoter Elements *In Vitro*. Fig. 3A: Diagram of construct for testing *in vitro* promoter activity. Arrows t_1 and t_2 denote the two transcripts terminating within the *his* (T*his*) and *thr* (T*thr*) attenuators. Fig. 3B Tobacco PrrnP1 promoter deletion derivatives. Nucleotide position is given relative to the transcription initiation site (+1; horizontal arrows). The position of the conserved -35 and -10 elements are marked. Plasmid names are listed on the right. Fig. 3C: Autoradiograph of the *in vitro* transcripts and relative quantities. Values were determined as described in Methods and are averages of three experiments.

Figures 4A and 4B show the results of scanning Mutagenesis for mapping PrrnP1 Promoter Elements *In Vitro*. Fig. 4A: DNA sequence of P*rrn* promoter derivatives (SEQ ID NOS: 3-30). Plasmid names with mutated nucleotide positions are listed on the left. On top a horizontal arrow marks the positions of multiple transcription initiation sites (TIS) and vertical

arrowheads mark the -64, +17 and +37 positions relative to the TIS marked +1. Relevant cloning sites are labeled. The conserved -35 and -10 promoter elements are boxed. Dots in the alignment represent identical sequences. Non-plastid nucleotides are in lower case, mutated nucleotides are in bold. Fig. 4B: Autoradiograph of the *in vitro* transcripts and relative quantities. The origin of the t_1 and t_2 transcripts is explained in Figure 3. Signals of transcripts derived from the three initiation sites do not resolve. Bars represents the sum of signals of the t_1 + t_2 transcripts relative to clone pJYS112 (100%). Figure 4B was obtained by merging two independently obtained data sets. Values for clones on the left (plasmids pJYS15 through pJYS124) and on the right (pJYS112 through pJYS183) were normalized for their own control (pJYS112; 100%; black bar). Values were determined as described in Methods and are averages of three experiments.

Figures 5A and 5B show the strategy used to assess the contribution of the -10 region to promoter strength. Fig. 5A: DNA sequence of wild-type and mutant -10 region in barley (Kim et al., 1999) SEQ ID NOS: 31-36) and tobacco (this study)(portions of SEQ ID NOS: 3, 21, 22 and SEQ ID NO: 37) plastid promoters. Fig. 5B: Autoradiograph of the *in vitro* transcripts and relative transcription activity of the tobacco PrrnP1 derivatives. Values were determined as described in Methods and are averages of three experiments.

Figure 6 depicts a model for Interaction of the PEP with the PrrnP1 Promoter. Fig. 6A: Factor-independent activation of PrrnB1 transcription. Depicted here is sigma interaction with the RUA—35 region. Note, however, that the actual subunit involved in the interaction may

be a different subunit. Identified in the Figure are the α (αCTD, αNTD), β, β', β'' and σ subunits, and the RUA, -35 and -10 promoter elements. Fig. 6B: Factor-dependent activation of PrrnP1 transcription by activator bound to RUA.

Figure 7 shows the alignment of the *trnV* and *rrn* Intergenic Region in the Plastid Genome of Monocot and Dicot Species. Data are shown for tobacco (*N. tabacum*, Nt; SEQ ID NO: 38), rice (*O. sativa*, Os; SEQ ID NO:39), maize (*Zea mays*, Zm; SEQ ID NO:40), spinach (*S. oleracea*, So; SEQ ID NO:41), carrot (*D. carota*, Dc; SEQ ID NO:42), Arabidopsis (*A. thaliana*, At; SEQ ID NO:43), soybean (*G. max*, Gm; SEQ ID NO:44) and pea (*P. sativum*, Ps; SEQ ID NO:45). The ends of the structural genes for *trnV* and *rrn* are bracketed. The RUA, -35 and -10 conserved promoter elements are boxed. Horizontal arrows mark transcription initiation sites from Pc, PrrnP1 (P1) and PrrnP2 (P2) promoters. Vertical arrows denote position of tobacco processing sites. Dashes represent gaps in the alignment. Conserved nucleotide positions are denoted by asterisk below the alignment.

Figure 8 shows block mutagenesis of nucleotides in the wild-type Prrn promoter (Prrn10; SEQ ID NO: 3) at neutral positions to minimize DNA sequence homology of Prrn promoters. The mutant derivative is Prrn11 (SEQ ID NO: 51). The sequences shown are suitable for combination with translation control sequences for transgene expression, as described in pending patent application WO 00/07421 for increasing protein expression levels.

Detailed Description of the Invention

Expression of the plastid rRNA operon (*rrn*) during development is highly regulated at the level of

transcription. The plastid *rrn* operon in most higher plant species is transcribed by the PEP, the multisubunit plastid RNA polymerase from PrrnP1, a sigma-70 type promoter with conserved -10 and -35 core promoter elements. To identify functionally important sequences, the tobacco PrrnP1 was dissected *in vivo* and *in vitro*. Based on the *in vivo* deletion analysis, sequences upstream of nucleotide -83 do not significantly contribute to promoter function.

The more detailed *in vitro* dissection described herein identified an RUA (rRNA upstream activator), a conserved 6 bp sequence directly upstream of the -35 core promoter element responsible for enhanced transcription from the PrrnP1 promoter core. Furthermore, the *in vitro* dissection revealed that the -35 hexamer, but not the -10 element, is crucial for promoter activity. Mutagenesis of sequences downstream of the transcription start site lead to enhanced *in vitro* transcription. We propose that sigma interaction with the -10 element in PrrnP1 is in part replaced by direct PEP-RUA (protein-DNA) interaction or by protein-protein interaction between the PEP and an RUA-binding transcription factor.

The following definitions are provided to aid in understanding the subject matter regarded as the invention.

"Heteroplastomic" refers to the presence of a mixed population of different plastid genomes within a single plastid or in a population of plastids contained in plant cells or tissues.

"Homoplastomic" refers to a pure population of plastid genomes, either within a plastid or within a population contained in plant cells and tissues. Homoplastomic plastids, cells or tissues are genetically stable because they contain only one type of plastid genome. Hence, they remain homoplastomic even after the

selection pressure has been removed, and selfed progeny are also homoplastomic. For purposes of the present invention, heteroplastomic populations of genomes that are functionally homoplastomic (i.e., contain only minor populations of wild-type DNA or transformed genomes with sequence variations) may be referred to herein as "functionally homoplastomic" or "substantially homoplastomic." These types of cells or tissues can be readily purified to a homoplastomic state by continued selection.

"Plastome" refers to the genome of a plastid.

"Transplastome" refers to a transformed plastid genome.

Transformation of plastids refers to the stable integration of transforming DNA into the plastid genome that is transmitted to the seed progeny of plants containing the transformed plastids.

"Selectable marker gene" refers to a nucleic acid sequence that upon expression confers a phenotype by which successfully transformed plastids or cells or tissues carrying the transformed plastid can be identified.

"Transforming DNA" refers to homologous DNA, or heterologous DNA flanked by homologous DNA , which when introduced into plastids becomes part of the plastid genome by homologous recombination.

An alternative type of transforming DNA refers to a DNA which contains recombination site sequences for a site-specific recombinase or integrase. Insertion of this type of DNA is not dependent on the degree of homology between the transforming DNA and the plastid to be transformed but rather is catalyzed by the action of the recombinase or integrase on the first and second recombination sites.

"Operably linked" refers to two different regions or two separate nucleic acid sequences spliced together in a construct such that both regions will function to promote gene expression and/or protein translation.

"Nucleic acid" or a "nucleic acid molecule" as used herein refers to any DNA or RNA molecule, either single or double stranded and, if single stranded, the molecule of its complementary sequence in either linear or circular form. In discussing nucleic acid molecules, a sequence or structure of a particular nucleic acid molecule may be described herein according to the normal convention of providing the sequence in the 5' to 3' direction. With reference to nucleic acids of the invention, the term "isolated nucleic acid" is sometimes used. This term, when applied to DNA, refers to a DNA molecule that is separated from sequences with which it is immediately contiguous in the naturally occurring genome of the organism in which it originated. For example, an "isolated nucleic acid" may comprise a DNA molecule inserted into a vector, such as a plasmid or virus vector, or integrated into the genomic DNA of a prokaryotic or eukaryotic cell or host organism.

When applied to RNA, the term "isolated nucleic acid" refers primarily to an RNA molecule encoded by an isolated DNA molecule as defined above. Alternatively, the term may refer to an RNA molecule that has been sufficiently separated from other nucleic acids with which it would be associated in its natural state (i.e., in cells or tissues). An isolated nucleic acid (either DNA or RNA) may further represent a molecule produced directly by biological or synthetic means and separated from other components present during its production.

The phrase "heterologous molecule" refers to a molecule which is produced in the plant following

introduction of a nucleic acid of the invention. Such molecules include RNA, (e.g., siRNA) and proteins.

The terms "percent similarity", "percent identity" and "percent homology" when referring to a particular sequence are used as set forth in the University of Wisconsin GCG software program.

The term "functional" as used herein implies that the nucleic or amino acid sequence is functional for the recited assay or purpose.

The phrase "consisting essentially of" when referring to a particular nucleotide or amino acid means a sequence having the properties of a given SEQ ID No:. For example, when used in reference to an amino acid sequence, the phrase includes the sequence per se and molecular modifications that would not affect the basic and novel characteristics of the sequence.

The phrase Prnn transcription modulating element (PTME) as used herein refers to elements identified in accordance with the invention and include the RUA, the -35 hexamer, the G-patch, -10 region, the spacer between the -10 and TIS and the ITS region. See Figure 4.

A "vector" is a replicon, such as a plasmid, cosmid, bacmid, phage or virus, to which another genetic sequence or element (either DNA or RNA) may be attached so as to bring about the replication of the attached sequence or element.

An "expression operon" refers to a nucleic acid segment that may possess transcriptional and translational control sequences, such as promoters, enhancers, translational start signals (e.g., ATG or AUG codons), polyadenylation signals, terminators, and the like, and which facilitate the production of a polypeptide coding sequence in a host cell or organism. Such expression signals may be combined such that

production of said polypeptide occurs transiently or is produced stably over the life of the cell.

The term "oligonucleotide," as used herein refers to primers and probes of the present invention, and is defined as a nucleic acid molecule comprised of two or more ribo or deoxyribonucleotides, preferably more than three. The exact size of the oligonucleotide will depend on various factors and on the particular application and use of the oligonucleotide.

The term "probe" as used herein refers to an oligonucleotide, polynucleotide or nucleic acid, either RNA or DNA, whether occurring naturally as in a purified restriction enzyme digest or produced synthetically, which is capable of annealing with or specifically hybridizing to a nucleic acid with sequences complementary to the probe. A probe may be either single stranded or double stranded. The exact length of the probe will depend upon many factors, including temperature, source of probe and use of the method. For example, for diagnostic applications, depending on the complexity of the target sequence, the oligonucleotide probe typically contains 15 25 or more nucleotides, although it may contain fewer nucleotides. The probes herein are selected to be "substantially" complementary to different strands of a particular target nucleic acid sequence. This means that the probes must be sufficiently complementary so as to be able to "specifically hybridize" or anneal with their respective target strands under a set of pre-determined conditions. Therefore, the probe sequence need not reflect the exact complementary sequence of the target. For example, a non complementary nucleotide fragment may be attached to the 5' or 3' end of the probe, with the remainder of the probe sequence being complementary to the target strand. Alternatively, non complementary bases or longer

sequences can be interspersed into the probe, provided that the probe sequence has sufficient complementarity with the sequence of the target nucleic acid to anneal therewith specifically.

The term "primer" as used herein refers to an oligonucleotide, either RNA or DNA, either single stranded or double stranded, either derived from a biological system, generated by restriction enzyme digestion, or produced synthetically which, when placed in the proper environment, is able to functionally act as an initiator of template-dependent nucleic acid synthesis. When presented with an appropriate nucleic acid template, suitable nucleoside triphosphate precursors of nucleic acids, a polymerase enzyme, suitable cofactors and conditions such as a suitable temperature and pH, the primer may be extended at its 3' terminus by the addition of nucleotides by the action of a polymerase or similar activity to yield an primer extension product. The primer may vary in length depending on the particular conditions and requirement of the application. For example, in diagnostic applications, the oligonucleotide primer is typically 15 25 or more nucleotides in length. The primer must be of sufficient complementarity to the desired template to prime the synthesis of the desired extension product, that is, to be able anneal with the desired template strand in a manner sufficient to provide the 3' hydroxyl moiety of the primer in appropriate juxtaposition for use in the initiation of synthesis by a polymerase or similar enzyme. It is not required that the primer sequence represent an exact complement of the desired template. For example, a non complementary nucleotide sequence may be attached to the 5' end of an otherwise complementary primer. Alternatively, non complementary bases may be interspersed within the oligonucleotide primer sequence,

provided that the primer sequence has sufficient complementarity with the sequence of the desired template strand to functionally provide a template primer complex for the synthesis of the extension product.

Amino acid residues described herein are preferred to be in the "L" isomeric form. However, residues in the "D" isomeric form may be substituted for any L amino acid residue, provided the desired properties of the polypeptide are retained. All amino acid residue sequences represented herein conform to the conventional left-to-right amino terminus to carboxy terminus orientation.

The term "tag," "tag sequence" or "protein tag" refers to a chemical moiety, either a nucleotide, oligonucleotide, polynucleotide or an amino acid, peptide or protein or other chemical, that when added to another sequence, provides additional utility or confers useful properties, particularly in the detection or isolation, to that sequence. Thus, for example, a homopolymer nucleic acid sequence or a nucleic acid sequence complementary to a capture oligonucleotide may be added to a primer or probe sequence to facilitate the subsequent isolation of an extension product or hybridized product. In the case of protein tags, histidine residues (e.g., 4 to 8 consecutive histidine residues) may be added to either the amino or carboxy terminus of a protein to facilitate protein isolation by chelating metal chromatography. Alternatively, amino acid sequences, peptides, proteins or fusion partners representing epitopes or binding determinants reactive with specific antibody molecules or other molecules (e.g., flag epitope, c myc epitope, transmembrane epitope of the influenza A virus hemaglutinin protein, protein A, cellulose binding domain, calmodulin binding protein, maltose binding protein, chitin binding domain,

glutathione S transferase, and the like) may be added to proteins to facilitate protein isolation by procedures such as affinity or immunoaffinity chromatography. Chemical tag moieties include such molecules as biotin, which may be added to either nucleic acids or proteins and facilitates isolation or detection by interaction with avidin reagents, and the like. Numerous other tag moieties are known to, and can be envisioned by, the trained artisan, and are contemplated to be within the scope of this definition.

As used herein, the terms "reporter," "reporter system", "reporter gene," or "reporter gene product" shall mean an operative genetic system in which a nucleic acid comprises a gene that encodes a product that when expressed produces a reporter signal that is a readily measurable, e.g., by biological assay, immunoassay, radioimmunoassay, or by colorimetric, fluorogenic, chemiluminescent or other methods. The nucleic acid may be either RNA or DNA, linear or circular, single or double stranded, antisense or sense polarity, and is operatively linked to the necessary control elements for the expression of the reporter gene product. The required control elements will vary according to the nature of the reporter system and whether the reporter gene is in the form of DNA or RNA, but may include, but not be limited to, such elements as promoters, enhancers, translational control sequences, poly A addition signals, transcriptional termination signals and the like.

The terms "transform", "transfect", "transduce", shall refer to any method or means by which a nucleic acid is introduced into a cell or host organism and may be used interchangeably to convey the same meaning. Such methods include, but are not limited to, transfection, electroporation, microinjection, PEG-fusion, biolistic bombardment and the like.

A "clone" or "clonal cell population" is a population of cells derived from a single cell or common ancestor by mitosis.

A "cell line" is a clone of a primary cell or cell population that is capable of stable growth in vitro for many generations.

The following Examples are provided to describe certain embodiments of the invention. They are not intended to limit the invention in any way.

EXAMPLE I

The following materials and methods are provided to facilitate the practice of the present invention.

Alignment of *rrn* Promoter Regions

E. coli and tobacco promoter comparison was made using the *E. coli* genomic sequences 4,163,793-4,163,947 (accession NC_000913) and tobacco plastid sequences 102,472-102,560 (accession Z00044). Plant plastid *trnV/rrn* intergenic region comparisons were made using the following sequences: rice, 91,065-91,301 (accession X15901); maize, 94,931-95,166 (accession X86563); spinach, 97,717-97,949 (accession AJ400848.1); Arabidopsis, 100,778-101,014 (accession AP000423); tobacco, 102531-102,763 (Accession Z00044); carrot, 317-550 (accession X78534); soybean, complement of 1477-1703 (Accession X07675); and pea, 70-333 (accession M30826). Sequence comparisons were made using the ClustalW program of the Sequence Interpretation tools section of GenomeNet.

Plasmids for *In Vitro* Assays

Plasmids for the *in vitro* assay were obtained by cloning PCR-amplified promoter derivatives (*Sac*I-*Eco*RI

fragments) into plasmid pKL23, a pBluescript KSII+ plasmid derivative carrying two bacterial transcription terminators downstream of suitable (*Sac*I-*Eco*RI) restriction sites (Liere and Maliga, 1999).

The promoter fragments were designed to have a *Sac*I site at 5'end and an *Eco*RI site at 3'end. The 5'ends correspond to the following nucleotides of the tobacco plastid genome (Wakasugi et al., 1998): P*rrn*-175, 102,475; P*rrn*-105, 102,542; P*rrn*-83, 102,564; P*rrn*-64, 102,583; P*rrn*-38, 102,609; P*rrn*+37, 102,683; P*rrn*+17, 102,663; and P*rrn*+12, 102,658. The endpoints of the 5' deletion at P*rrn*-175 encoded part of the cloning site *Sac*I, whereas endpoints of the 3' deletions P*rrn*+37, P*rrn*+17 and P*rrn*+12 encoded part of the *Eco*RI site. For scanning mutagenesis, 3bp mutations were incorporated in the respective PCR primers. The DNA sequence of the promoter derivatives was confirmed by sequencing.

Plastid Transformation Vectors

Plasmid pPS205 contains a chimeric *uidA* reporter gene as a *Sac*I-*Hind*III fragment in a pPRV111A plastid vector (Figure 2). Plasmid pPS205 is a plasmid pPS6 derivative (Sriraman et al., 1998b). The chimeric *uidA* gene consists of: Between the *Sac*I and *EcoRI* sites, the test promoter fragment PrrnP1 -83/+37 with +1 being the transcription start site (Figure 1); Between the *EcoRI* and the *Nco*I sites, a synthetic ribosome binding site with the following sequence 5'-CTCGAGAATTCAGTT GTAGGGAGGGATCCATGG-3' SEQ ID NO: 46; Between the *Nco*I and *Xba*I sites, the *uidA* coding region with an N-terminal c-myc tag corresponding to amino acids 410-419 (EQKLISEEDL; SEQ ID NO: 47) within the carboxy terminal domain of the human c-*myc* protein; Between the *Xba*I and *Hind*III sites the 3' untranslated region of the *rps16* ribosomal protein gene (Trps16). The *Sac*I-*EcoRI* promoter fragment of pPS205

was replaced with PrrnP1 -38/+37 to obtain pPS206, with PrrnP1 -175/+37 to obtain pPS207 and with PrrnP1 -105/+37 to obtain pPS208. The *SacI-EcoRI* fragments for plasmids pPS205 through pPS208 were obtained by PCR amplification as described for the construction of *in vitro* test plasmids.

***In vitro* Transcription Assay**

High salt extracts were prepared from Percoll step gradient-purified chloroplasts of young leaves of 6-8 week old tobacco (*Nicotiana tabacum* cv. Petit Havana) plants (Orozco et al., 1986). The final ammonium sulfate pellets were resuspended at a ratio of 1 ml DEAE buffer per 7.5 mg of chlorophyll. For the *in vitro* transcription reaction, supercoiled plasmid DNA (0.9376 pmole) was incubated at 30°C for 20 min in a 20 µl reaction mix consisting of 8.0 µl plastid protein extract (equiv.~9.0 x 10^7 chloroplasts), 12 mM HEPES-KOH pH 8.0, 10 mM $MgCl_2$, 40 mM KCl, 10 mM DTT, 500 µM each of GTP and CTP and 50 µM each of ATP and UTP and 5.4 u of RNA guard (Amersham Biosciences, Piscataway, NJ), 17.21 - 24.25 µCi of α^{32}P-UTP (6000 Ci·mmole^{-1}; Perkin Elmer Life Sciences, Boston, MA) was included to allow detection and quantitation of transcribed products. The reactions were stopped with the addition of 115 µl of RNA extraction mix (0.36 M NaCl, 20 mM EDTA, 10 mM Tris pH 8 and 1% SDS), 15 µl of 5 M NH_4OAc, 40 µg of yeast tRNA, and extracted with a 1:1 mix of phenol and chloroform. 120 µl of the supernatant was precipitated with 150 µl isopropanol. The final pellet was resuspended in 6 µl of loading dye (Ambion, Austin, TX) and half was loaded on a 6.0% Long Ranger gel (BioWhittaker Molecular Applications, Rockland, ME). Relative transcript levels were quantitated using a PhosphorImager and the ImageQuant program (Amersham

Biosciences, Piscataway, NJ) with values normalized. The Phosphoimager values of t_1 and t_2 were first individually normalized for background signals from each lane, divided by the number of predicted U residues (47 and 59, respectively) in their transcripts and then subsequently added together.

Plastid Transformation

Plastid transformation and characterization of transplastomic lines was carried out as described (Svab and Maliga, 1993).

RNA Gel Blot Analysis

Total cellular RNA was extracted from plants grown in sterile culture (MS salts, 3% sucrose, 0.7% agar; 18 hr light and 6 hr dark cycle) by the method of Stiekema (Stiekema et al., 1988). RNA (1 μg per lane) was separated in 1.2% agarose, formaldehyde-MOPS gels and blotted by the Posiblot transfer apparatus (Stratagene, La Jolla, CA) onto Hybond N membranes (Amersham Biosciences, Piscataway, NJ). The 25S rDNA probe was PCR amplified using primers '5-TCACCTGCCGAATCAACTAGC-3' (SEQ ID NO: 48) and '5-GACTTCCCTTGCCTACATTG-3' (SEQ ID NO: 49) and total tobacco cellular DNA as template (Dempsey et al., 1993). The *uidA* probe was the *NcoI/XbaI* fragment from plasmid pPS6 (Sriraman et al., 1998b). Radioactive probes were prepared using the Ready-To-Go DNA labeling beads (Amersham Biosciences, Piscataway, NJ) and α^{32}P-dCTP. Blots were hybridized at 65°C in Rapid Hybridization buffer (Amersham Biosciences, Piscataway, NJ).

Upon request, all novel material described in this publication will be made available in a timely manner for non-commercial research purposes.

Examination of the *rrn* Upstream Region for Potential Regulatory Sequences

Promoter elements regulating transcription, at least in the case of the plastid *psbD* promoter are localized upstream of the -35 promoter core (Allison and Maliga, 1995; Kim and Mullet, 1995; Kim et al., 1999; Thum et al., 2001). Therefore, we have searched for potential regulatory elements between the plastid *trnV* gene and the *rrn* coding region. Given conservation of the *E. coli* and plastid PEP transcription machineries, we used the well-characterized *E. coli rrn*B P1 promoter regulatory sequences as a guide (Figure 1)(Ross et al., 1993).

Two types of *cis* elements are responsible for the *E. coli* rrnB P1 promoter strength. One is the UP element, a 20 bp AT-rich region directly upstream (-40 to -60) of the promoter core. The UP element interacts directly with the *E. coli* RNA polymerase α subunit C-terminal domain increasing the basal promoter activity by 30-60 fold (Ross et al., 1993; Rao et al., 1994; Aiyar et al., 1998; Gourse et al., 2000). The tobacco *rrn* P1 lacks an AT-rich sequence in the region corresponding to the *E. coli* UP element. A second type of *E. coli cis* regulatory element is the Fis binding site. Fis is a 11.2kDa DNA binding, DNA bending, highly conserved protein in bacteria originally identified as Factor for inversion stimulation (Ross et al., 1990). The spacing between the Fis binding sites differs among the 7 ribosomal RNA P1 promoters of *E. coli*, yet all contribute albeit to different extents to promoter activity (Hirvonen et al., 2001). The positions of the *E. coli* Fis binding sites were considered when designing the deletion endpoints for the *in vivo* PrrnP1 promoter analysis.

Dissection of the PrrnP1 Upstream Region *in vivo*

In vivo testing of promoter derivatives is the most reliable approach to identify promoter elements. Thus, we prepared an ordered set of PrrnP1 promoter deletion derivatives that were fused with the *uidA* reporter gene (Figure 2A). The reporter genes were cloned into a plastid transformation vector in which *uidA* is linked to a selectable spectinomycin resistance (*aadA*) gene. The transgenes were then introduced into the tobacco plastid genome. Four transplastomic lines were obtained in which *uidA* is expressed from a PrrnP1 derivative (Figure 2A).

RNA gel blot analysis was carried out to determine *uidA* mRNA accumulation in the leaves of the transgenic plants. Results shown in Figure 2 indicate that deletion of the -175 to -83 region has no effect on *uidA* transcription. However, deletion of nucleotides between -83 to -38 completely eliminated promoter activity as no signal could be detected even on over-exposed films (data not shown). Thus, based on the *in vivo* deletion analysis, there are no PrrnP1 promoter elements upstream of nucleotide -83.

Dissection of the PrrnP1 Upstream Region *in vitro*

In vivo promoter analysis is very labor intensive, therefore, we decided to define the PrrnP1 upstream promoter region *in vitro*. As the first step, we duplicated the *in vivo* upstream deletion analyses *in vitro*, and included downstream deletions to define a smaller, fully functional PrrnP1 that is suitable for scanning mutagenesis. Promoter activity was determined by measuring RNA accumulation from mini-genes, which consist of a PrrnP1 promoter derivative and two ρ-independent bacterial attenuators that function as transcription terminators *in vitro* (Chen et al., 1990; Liere and

Maliga, 1999)(Figure 3A). *In vitro* transcription was performed in crude, high salt extracts of purified chloroplasts allowing multi-round transcription during a defined period.

The consequence of deleting sequences upstream of the promoter core was tested on PrrnP1 derivatives with nucleotide +37 at the 3'-end (Figure 3B). Quantitation of the *in vitro* transcripts from the PrrnP1 promoter 5' deletion clones is consistent with the *in vivo* results: sequences between -175 to -83 have no significant effect on promoter activity. The 5'-deletion series included one additional construct not tested *in vivo*, deletion of sequences between -83 and -64 (pJYS112), which also had no significant affect on transcription. Deletion of sequences between nucleotides -64 and -38 reduced transcript accumulation by five-fold. Deletion of the conserved -35 promoter element practically abolished *in vitro* transcription activity (plasmid pJYS111, Figure 3B and 3C).

The consequence of deleting sequences downstream of the promoter core was tested on PrrnP1 derivatives with nucleotide -64 at the 5'-end (Figure 3B). The 3'-end was shortened in two steps, to +17 (pJYS194)and +12 (pJYS195). Data on *in vitro* transcript accumulation indicate that sequences between -64 and +17 are sufficient for full PrrnP1 promoter activity.

Scanning Mutagenesis to Define PrrnP1 Promoter Architecture *in Vitro*

Deletion analysis is suitable to define promoter boundaries. However, individual elements within a promoter can be best defined by systematically changing blocks of sequences within a larger DNA fragment. Therefore, we performed a 3-bp scanning mutagenesis of

the full PrrnP1 promoter (-64/+17) defined by deletion analysis *in vitro*. The point mutations and transcription activity of the promoters are shown in Figure 4.

In the region upstream of the -35/-10 promoter core mutations significantly (3 to 5 fold) reduced transcription in two clones with mutations in the -43 to -38 region (pJYS120, pJYS121). Point-mutations in the conserved GTGGGA sequence reduced transcription activity to the same extent as deletion of the entire sequence upstream of the conserved -35 promoter element (sequences upstream of -38 position, construct pJYS12, Figure 3). Since the conserved hexamer is required for PrrnP1 promoter strength, it is designated the plastid rRNA upstream activator (RUA).

Mutagenesis of the core promoter region (nucleotides -37 to -8) significantly affected transcription only in the conserved -35 (TTGACG) promoter element. Mutagenesis of TTG practically abolished (1.77%), whereas mutagenesis of ACG severely reduced (to 8.19%) transcription, confirming the importance of the -35 promoter element in PrrnP1 promoter recognition. However, mutations including the -10 promoter element (-16/-8 region, plasmids pJYS173, pJYS174, pJYS175) only moderately reduced (to 70-80%) *in vitro* transcription. Mutagenesis of the G-rich sequence (G-patch) between nucleotides -28 to -23 also reduced transcription activity by ~30% (pJYS169, pJYS170) (Figure 4). Interestingly, mutation in sequences downstream of the transcription initiation site (TIS) from +9 to +14 (pJYS181, pJYS182) caused an increase in transcription activity of at least 2 fold.

To directly address the role the -10 sequence, the first and last T of the hexamer was mutated to A, as these mutations essentially abolished transcription from the *psbD*, *rbcL* and *psbA* promoters (Kim et al., 1999). These mutations reduced PrrnP1 activity only to 46%,

confirming a relatively limited role for the -10 region in PrrnP1 recognition by the PEP (Figure 5).

Ribosomal RNA Upstream Activating Sequence

In this study we have identified a conserved hexameric sequence, GTGGGA (SEQ ID NO: 50), the rRNA operon RUA element directly upstream of the -35 box, as an essential sequence required for overall PrrnP1 promoter activity. Apparently, RUA is the only element upstream of the promoter core. Based on *in vivo* deletion analysis, sequences upstream of nucleotide -83 do not significantly contribute to promoter function. The *in vitro* analyses then pinpointed RUA as the source of promoter strength. Thus PrrnP1, as all characterized plastid promoters, is remarkably compact, lacking regulatory sequences far upstream or downstream of the -35/-10 promoter core. The only exception is the blue-light regulated *psbD* promoter: the AAG box is located between -36 to -64 and the PGT-box is located between -71 to -100, respectively (Allison and Maliga, 1995; Kim and Mullet, 1995; Kim et al., 1999; Thum et al., 2001). The *E. coli* rrnB P1 promoter has two distinct sets of regulatory elements: the UP element (-40 to -60) that is part of the promoter recognition domain and Fis binding sites (-64 to -150). In contrast, the plastid promoter core appears to lack regulatory regions that extend far upstream from the core.

It is possible that the RUA directly interacts with a component of the PEP itself in which case the RUA acts as an extension of the promoter core facilitating binding of the PEP and enhancing promoter strength. In this case the plastid RUA would play a role similar to the *E. coli rrnB* P1 UP element which is responsible for increasing promoter strength by direct interaction with RNA polymerase α subunit C-terminal domains (Ross et al.,

1993). RUA could be recognized by a plant sigma factor in a manner in which the extended -10 element is recognized by bacterial σ^{70} factors (Bown et al., 1997)(Figure 6A). Various plant sigma factors have been shown to exhibit promoter preference *in vitro* (Tiller and Link, 1993) (Hakimi et al., 2000)and *in vivo* (Kanamaru et al., 2001) and exhibit organ-specific, light-induced, circadian and developmentally regulated expression patterns (Isono et al., 1997; Tanaka et al., 1997; Kestermann et al., 1998; Tozawa et al., 1998; Lahiri et al., 1999; Morikawa et al., 1999; Tan and Troxler, 1999; Fujiwara et al., 2000; Lahiri and Allison, 2000; Tsunoyama et al., 2002). Thus, an interaction of the RUA with a specific sigma factor(s) could be the means to selectively regulate transcription of the tobacco rRNA operon. Direct interaction of RUA with the PEP would be a factor-independent mechanism to enhance *rrn* transcription. The alternative factor-dependent mechanism would involve a nuclear-encoded, plastid-targeted factor that would bind to the RUA and facilitate binding of the PrrnP1 promoter by the PEP (Figure 6B).

Preliminary analysis of the PrrnP1 promoter has been reported in pea (Sun et al., 1989). Since transcription was carried out with linear DNA, a poor template for the PEP, the pea data are not directly comparable to our results.

The Role of the Conserved -10 Promoter Element in PrrnP1 Function

PrrnP1 contains the conserved -35 (TTGACG) and -10 (TATATT) promoter elements that are a variant of the sigma 70-type consensus sequence (TTGaca and TataaT, respectively) obtained for plastid promoters (Link, 1994). Mutagenesis of either of the conserved promoter

elements is expected to abolish promoter activity (Sugiura, 1992; Gruissem and Tonkyn, 1993; Link, 1994, 1996; Liere and Maliga, 2001; Kim, 1999). Transcription from the *psbA* and *psbD* promoters, at least in some species and at certain developmental stages, is dependent only on the -10 promoter element. Interaction of the PEP with the -35 element is probably replaced by interaction with the extended -10 sequence (*psbA* promoter in wheat) (Satoh et al., 1999), the TATA-like sequence between the -35/-10 elements (*psbA* promoter in mustard) (Eisermann et al., 1990) or factors binding to sequences upstream of a degenerate -35 element (*psbD* promoter in barley and tobacco) (Allison and Maliga, 1995; Kim and Mullet, 1995; Kim et al., 1999; Thum et al., 2001). To date, PrrnP1 is the first plastid promoter in higher plants in which the -10 sequence seems to play a minor role. Mutagenesis of the -10 sequence, and of the three nucleotides directly upstream, only slightly reduced transcription activity *in vitro* (by 20% to 40%; pJYS173,pJYS174, pJYS175; Figure 4). Mutating the first and last T of the -10 hexamer to an A reduced PrrnP1 activity to 46% (Figure 5). In contrast, the same mutations had a much more dramatic affect on transcription of the strong *rbcL*, *psbD* and *psbA* promoters (Kim et al., 1999). We propose that sigma interaction with the -10 element is largely replaced by direct PEP-RUA (protein-DNA) interaction or by protein-protein interaction between the PEP and the RUA-binding transcription factor (see above).

Initial Transcribed Sequence Affects Transcription Efficiency

Mutations between sequences +9 to +14 (pJYS181, pJYS182) cause a ~2-fold increase in transcription activity (Figure 4). This region corresponds to the

Initial Transcribed Sequence (ITS; +1 to +20) of *E. coli* promoters. The phenomenon of enhanced transcript accumulation due to mutagenesis of the PrrnP1 +9 to +14 sequences is reminiscent of enhanced transcript accumulation from strong *E. coli* promoters due to mutagenesis of the ITS region (Chan and Gross, 2001). Strong promoter contacts in *E. coli* were shown to promote RNA polymerase binding, but impede promoter escape with a concomitant increase in the number of abortive short RNA products that form in the open complex of the RNA polymerase. This abortive initiation phase ends when the RNA polymerase moves away from the promoter and full-size transcripts are formed. Sequences within the ITS region were found to affect the frequency of promoter escape and formation of "productive" transcripts, that explains enhanced transcript accumulation due to mutagenesis of the PrrnP1 ITS region.

G-patches: An Unusual Feature of the PrrnP1 Promoter

The GC content of the plastid genome is relatively low, 37.85% (GenBank Accession No. Z00044). A striking feature of the tobacco PrrnP1 promoter is the preponderance of conserved G residues between the -35 and -10 promoter elements and in the region downstream of the promoter core (Figure 7). The cognate regions of the promoters of other highly expressed plastid genes, *rbcL*, *psbA* and *psbD* tend to be AT-rich. Mutagenesis of these G-patches only slightly reduced transcript accumulation *in vitro* (mutants pJYS169, pJYS170, pJYS177; Figure 4). However, the G-patches may have a role at specific stages during development similar to the role of TATA-like sequences in *psbA* transcription (Eisermann et al., 1990).

Conservation of PrrnP1 Promoter Elements

An alignment of the *trnV* and rRNA operon intergenic region for tobacco, rice, maize, spinach, carrot, Arabidopsis and pea is shown in Figure 7. Position of the conserved RUA, -35 and -10 PrrnP1 promoter elements and transcription initiation sites are marked. The RUA, -35 and -10 promoter elements are conserved in each of the species, except pea, suggesting a shared mechanism for the regulation of rRNA transcription in monocots and dicots. Interestingly, RUA is also present in spinach, a species in which PrrnP1 is not recognized as a promoter (Iratni et al., 1994; Iratni et al., 1997; Bligny et al., 2000). Conservation of a functional PrrnP1 promoter in spinach has been confirmed by showing that it is faithfully recognized by the PEP in tobacco plastids when driving expression of a reporter gene. Therefore, absence of a PrrnP1-specific transcription factor has been proposed as the reason for the lack of transcription from this promoter in spinach (Sriraman et al., 1998a). This explanation is compatible with factor-dependent activation of transcription from the PrrnP1 promoter. The alternative, factor-independent activation mechanism would imply species-specific differences in the PEP subunit interacting with the RUA sequence. Transcription from the PrrnP1 and Pc promoters in *Arabidopsis* chloroplasts is not mutually exclusive as both promoters are simultaneously recognized (Sriraman et al., 1998a). Thus, Arabidopsis is different from spinach, in which only Pc is utilized (Iratni et al., 1994; Iratni et al., 1997; Bligny et al., 2000).

Pea is the only species in the alignment in Figure 7 in which the GTGGGA RUA sequence is poorly conserved: there is insertion of a G between the RUA and -35 element and the first two nucleotides of the hexamer are altered.

Thus, pea has the taGGGAg sequence instead of GTGGGA sequence upstream of the -35 element. Nevertheless, transcription of the rRNA operon in pea is from the PrrnP1 promoter (Sun et al., 1989). For comparison, we have included the sequence of another legume, soybean, in which the RUA element is conserved but there are point mutations in both the -35 and -10 promoter elements relative to the other species. Transcript 5'-ends upstream of the soybean *rrn* operon have not been mapped. Given all the variations from the *rrn* promoters of other dicot species, it is possible that the legumes have developed yet another unique promoter variant for plastid *rrn* transcription.

EXAMPLE II

In the previous example, we have demonstrated that a conserved hexameric sequence directly upstream of the -35 hexameric element significantly contributes to PrrnP1 promoter strength. This RUA element may be combined with other plastid core promoters to favorably enhance promoter strength. Since the RUA sequence is present in a large number of species, these chimeric promoters may be utilized to advantage in biotechnological applications. The data reveal additional regions of Prrn that appear to affect promoter function. These are referred to as Prrn transcription modulating elements (PTME) and include the RUA, the -35 hexamer, the G-patch, -10 region, the spacer between the -10 and TIS and the ITS region (Figure 4). The Prrn promoter is exceptional. For example, the *rbcL* (Shinozaki and Sugiura, 1982; Shiina et al., 1998) and *atpE* (Kapoor et al., 1994) promoters comprise -35 and -10 promoter elements only. Addition of one or more of the aforementioned PTME to other core promoters known in the art should beneficially alter promoter strength and developmental timing of plastid gene expression. When

combining these PTME with other core promoter sequences, it is desirable to approximate the sequence spacing of the elements observed in the wild type Prrn promoter. This entails aligning the -35 and -10 regions of such promoters and operably linking at least one PTME as desired. Particularly useful for obtaining high levels of transcription is the combination of the RUA, -35 and G-patch of Prrn with the -10 and sequences between the -10 and the transcription initiation site.

The data presented herein provide useful information for the design of improved Prrn promoters, which are less prone to undergo homologous recombination with the plastid genome. The strong Prrn promoter is the preferred choice for the expression of recombinant proteins in chloroplasts (Maliga, 2003). Since the Prrn promoter is naturally present upstream of the plastid rRNA operon, insertion of transgenes with the Prrn promoter duplicates the Prrn promoter region. Loop-out via directly repeated sequences may lead to the loss of transgenes, an approach that is utilized for the selective removal of marker genes from the plastid genome (Iamtham and Day, 2000). The minimal Prrn promoter defined here is smaller than promoter fragments used in earlier work (US Patents 5,877,402 and 6,388,168) reducing the opportunity for homologous recombination which is desirable for certain applications. Furthermore, we have determined that mutations at certain nucleotide positions do not affect promoter function. These nucleotides can be combined to further reduce the probability of plastid genome rearrangements by homologous recombination. An example for combining neutral mutations in a Prrn promoter derivative is Prrn11 (SEQ ID NO: 51) shown in Figure 8.

US Patent 5,877,402 provides a number of plastid promoter sequences which are suitable for combination with the PTME of the present invention. As mentioned

previously, translation control sequences which enhance translation of heterologous proteins in higher plants are described in WO/00070421 and are also useful when operably linked to the PTME of the invention. Table 1 below provides a list of promoters suitable for combination with the PTME of the invention.

Table 1. PEP promoters

atpB (-611, 502, 488, -255) atpI (-130) clpP (-95)	US Patent 6,472,586 and cited references
psbA, rbcL, psbD	US Patent 5,877,402; Liere and Maliga (2001); and references cited
rrnB, trnQ, trnH, trnK, trnG, trnS, psbA, rps16, psbK	Link, 1994
atpE	Kapoor et al. 1994

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APENDIX I

Figure 1

Seq. ID No. 2 *E. coli* rRNA operon upstream region

GCT**GAACAATTATTGCCCGTTTTACAGCGTTACGGCTTCGAAAC**GCTCGAAAAACTGGCAGTTTTAGGCTGATTT**GGTTGAATGTTGCGC**GGTCAGAAAATTATTTT
AAATTTCCTC TTGTCAGGCCGGAATAACTCCCTATAATGCGCCACCACT

Seq. ID No. 1 *Nicotiana tabacum* rRNA operon upstream region

CTCAGCGGTAGAGTGTCACCTTGACGTGGTGGAAGTCATCAGTTCGA GCCTGATTATCCCTAAGCCCAATGTGAGTTTTTCTAGTTGGATTTGCTC
CCCCGCCGTCGTTCAATGAGAATGGATAAGAGGCTCGTGGGA TTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGA

Figure 4

	Seq ID No
pJYS112 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	3
pJYS113 gagctc**tac**AGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	4
pJYS114 gagctcATG**tct**ATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	5
pJYS115 gagctcATGAGA**tac**GATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	6
pJYS116 gagctcATGAGAATG**cta**AAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	7
pJYS117 gagctcATGAGAATGGAT**ttc**AGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	8
pJYS118 gagctcATGAGAATGGATAAG**tcc**CTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	9
pJYS119 gagctcATGAGAATGGATAAGAGG**gag**GTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	10
pJYS120 gagctcATGAGAATGGATAAGAGGCTC**cac**GGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	11
pJYS121 gagctcATGAGAATGGATAAGAGGCTCGT**gct**TTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	12
pJYS122 gagctcATGAGAATGGATAAGAGGCTCGTGGG**aac**ACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	13
pJYS123 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTG**tgc**TGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	14
pJYS124 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGAC**act**GGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	15
pJYS169 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGA**ccc**GGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	16
pJYS170 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGG**ccg**AGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	17
pJYS171 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGC**tcc**GATGGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	18
pJYS172 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGG**cta**GGCTATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	19
pJYS173 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGAT**ccg**TATATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	20
pJYS174 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGC**ata**ATTTCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	21
pJYS175 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTAT**taa**TCTGGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	22
pJYS176 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATAT**aga**GGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	23
pJYS177 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTC**ccc**AGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	24
pJYS178 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGG**tcg**GAACTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	25
pJYS179 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGC**ctt**CTCCGGGCGAATATGAAGCGCATGGATACAAGaattc	26
pJYS180 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAA**gag**CGGGCGAATATGAAGCGCATGGATACAAGaattc	27
pJYS181 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTC**gcc**GCGAATATGAAGCGCATGGATACAAGaattc	28
pJYS182 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGG**cgc**AATATGAAGCGCATGGATACAAGaattc	29
pJYS183 gagctcATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAACTCCGGGCG**tta**ATGAAGCGCATGGATACAAGaattc	30

Figure 5

		Seq. ID No.
PpsbA	TTGACATTGGTATATAGTCTATGTTATACT	31
PpsbA mutant	TTGACATTGGTATATAGTCTATGTaATACa	32
PrbcL	TTGCGCTATACCTATCAAAGAGTATACAAT	33
PrbcL mutant	TTGCGCTATACCTATCAAAGAGTAaACAAa	34
PpsbD	AAAGAAGCATAAAGTAAGTAGACCTGACTCCTTGAATGATGCCTCTATCCGCTATTCT	35
PpsbD mutant	AAAGAAGCATAAAGTAAGTAGACCTGACTCCTTGAATGATGCCTCTATCCGCaATTCa	36

PJYS112	GTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGA	3
PJYS174	GTGGGATTGACGTGAGGGGGCAGGGATGGCataATTTCTGGGAGCGA	21
pJYS175	GTGGGATTGACGTGAGGGGGCAGGGATGGCTATtaaTCTGGGAGCGA	22
pJYS199	GTGGGATTGACGTGAGGGGGCAGGGATGGCaATATaTCTGGGAGCGA	37

Figure 7

N. tabacum SEQ ID NO: 38

CTAAGCCCAATGTGAGTTTTTCTAGTTGGATTTGCTCCCCCGCCGTCGT
TCAATGAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCT

GGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGTTATGCCTTG

GAATGAAAGACAATTCCGAATCCGCTTTGTCTACGAACAAGGAAGCTATAAGTAATGCAACTATGAATCT

O. sativa SEQ ID NO: 39

CTAAACCCAATGTGAGTTTTTTCTATTTTGACTTACTCCCCCCGCCACGAT
CGAACGGGAATGGATAAGAGGCTTGTGGGATTGACGTGATAGGGTAGGGTTGGCTATACTGCT

GGTGGCGAACTCCAGGCTAATAATCTGAAGCGCATGGATACAAGTTATCCTTG

GAAGGAAAGACAATTCCGAATCCGCTTTG
TCTACGAATAAGGAAGCTATAAGTAATGCAACTATGAATCT

Z. mays SEQ ID NO: 40

CTAAACCTAATGTGAGTTTTTTCTATTTTGACTTACTCCCCCACCACGAT
CGAACGGGAATGGATAGGAGGCTTGTGGGATTGACGTGATAGGGTAGGGTTGGCTATACTGCT

GGTGGCGAACTCCAGGCTAATAATCTGAAGCGCATGGATACAAGTTATCCTTG

GAAGGAAAGACAATTCCGAATCCGCTTTG
TCTACGAATAAGGAAGCTATAAGTAATGCAACTATGAATCT

S. oleracea SEQ ID NO: 41
CTAAACCCAACGTCAGTTTTTCTATTTTGACTTGCTCCCCCGCCGTGAT
TGAATGAGAATGAATAAGAGGCTCGTGGGATTGACGTGAGGGGGTAGGGATGGCTATATTTCT

GGGAGCGAACTCCAGGCGAATATGAAGCGCATGGATACAAGTTATGCCTTG

GAATGAAAGACAATTCCGAATCCGCTTTG
TCTACGAACAAGGAAGCTATAAGTAATGCAACTATGAATCT

D. carota SEQ ID NO: 42
CTAAATCCCAATGGGAGTTTTTCTATTTTGATTTGCTCCCCCGCCGTGAT
TGAACGAGAATCAAGAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCT

GGGAGCGAACTCCGGGCGAATATGAAGCGCATGGATACAAGTTAGGCCTTG

GAATGAAAGACAATTCCGAATCCGCTTTG
TCTACGAACAAGGAAGCTATAAGTAATGCAACTATGAATCT

A. thaliana SEQ ID NO: 43
CTAAACCCAATGAATGTGAGTTTTTCTATTTTGACTTGCTCCCTCGCTGTGAT
CGAATAAGAATGGATAAGAGGCTCGTGGGATTGACGTGAGGGGGTAGGGGTAGCTATATTTCT

GGGAGCGAACTCCATGCGAATATGAAGCGCATGGATACAAGTTATGACTTG

GAATGAAAGACAATTCCGAATCAGCTTTG
TCTACGAAGAAGGAAGCTATAAGTAATGCAACTATGAATCT

G. max SEQ ID NO: 44
CTAAACCCAATGTAAGTTTTTCTATTTGTATGCCGTGATCGAATAATAAT
TGAGAATGGATAAGAGGCTCGTGGGATTACACGAGGGGTGGGGGGGCTATATTTCT

GGGAGCGAACTCCAGTCGAATATGAAGCGCCTGGATACAAGTTATGCCTTG

GAATGGAAGAGAATTCCGAATCAGCTTTG
TCTACGAACAAGGAAGCTATAAGTAATGCAACTAGGAATCT

P. sativum SEQ ID NO: 45

CTAAACCCAATATCAATTTTTCCATTTGAGGACGAGAT
CCAATCTGAGTAGATAAGAGGATAGGGAGTTGACACAAGGGGGGGTAAGGCCATATAATATT
TATGGGAGGCAACTCCGGGCGAATAGTAAGCCCATGGATACAAGTCAAGTTATGTCTTCT
CAGTTCAGTAACTGAAATCAAATTTAAGTTCAGTAAATGAAATCAAATTCCGAATCAGCTTTG
TCTAGAAACAAGGAAGCTATAAGTAATGCAACTAGGAAGCT

Figure 8

Wt Prrn

AAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGCGAA SEQ ID NO: 3

Prrn10
gagctcAAGAGGCTCGTGGGATTGACGTGAGGGGGCAGGGATGGCTATATTTCTGGGAGGCGAA SEQ ID NO: 3

Prrn11

gagctcttctccgagGTGGGATTGACGTGAGGGGGCtccctaGGCTATATTagaGGGAGCGAA SEQ ID NO: 51

While certain of the preferred embodiments of the present invention have been described and specifically exemplified above, it is not intended that the invention be limited to such embodiments. Various modifications may be made thereto without departing from the scope and spirit of the present invention, as set forth in the following claims.